PRODUCTION OF XYLITOL BY *CANDIDA KRUSEI* USING XYLOSE FROM OIL PALM EMPTY FRUIT BUNCH (OPEFB) AS A SUBSTRATE

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NUR PUTIH IKHRAM ILANI BINTI ALIAS

Thesis submitted in partial fulfilment of the requirements
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APRIL 2013

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature	:
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Date	: 28TH JUNE 2013

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature :
Name : NUR PUTIH IKHRAM ILANI BINTI ALIAS
ID Number : KE10062
Date : 28TH JUNE 2013

Dedicated especially to my beloved mother, my father, siblings, lecturers, friends and to those who gives me support and inspiration that made this work possible.

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ABSTRACT

Xylitol, is extensively used in food, pharmaceutical and thin coating applications. The most important application of xylitol is its use as an alternative sweetener in foods for diabetic patients. Besides that, xylitol also important are as an anticariogenic agent in tooth paste formulations, as thin coatings on chewing vitamin tablets, in mouth washes, beverages and in bakery products (Rahman *et al*., 2006). There are few ways to gain this product. One of the ways is by using alkali extraction process. In this process, the main important reagent is Sodium Hydroxide (NaOH-5%) which will be used to gain the desire product (Sun *et al*., 1999).

Besides that, the other method that can be used to get this product is by using acid hydrolysis and as this process is simpler, and this experiment will be done by using this acid hydrolysis method to obtain the product. By using acid hydrolysis process, the EFB will put in contact with the acid and as the reaction occur, xylose will be produced. The product obtained then will be filtrate to separate the solution with the insoluble EFB during the experiment (Rahman *et al*., 2006). In order to produce xylitol from xylose, fermentation process using suitable microorganism is required. Therefore, xylose will undergo fermentation process by using *Candida krusei* a type of yeast which can convert xylose to xylitol after certain period time with suitable culture media (Mohamad *et al*., 2009).

There are three parameters that were used in this experiment which are the effect of time of hydrolysis, effect of pH and effect of amount of hydrolysate to the production of xylitol. From the result obtained it was found that the duration time for acid hydrolysis was found to be 150 minutes, the pH was 2 and the value of the hydrolysate was found to be 50 mL. All these parameter value lead to the high production of xylitol.

ABSTRAK

Xylitol, digunakan secara meluas dalam makanan dan farmaseutikal. Penggunaan yang paling penting adalah penggunaannya sebagai pemanis alternatif dalam makanan untuk pesakit kencing manis. Selain itu, Xylitol juga penting sebagai agen anticariogenic dalam rumusan ubat gigi, lapisan nipis pada tablet vitamin, dan sebagai bahan dalam pencuci mulut, minuman dan produk roti (Rahman et al., 2006). Terdapat beberapa cara untuk menghasilkan produk ini. Salah satu cara adalah dengan menggunakan proses pengekstrakan alkali. Dalam proses ini, reagen penting utama adalah Natrium Hidroksida (NaOH-5%) yang akan digunakan untuk mendapatkan produk yang diinginkan (Sun et al., 1999).

Selain itu, kaedah lain yang boleh digunakan untuk mendapatkan produk ini adalah dengan menggunakan cara hidrolisis asid dan kerana proses ini adalah lebih mudah. Dengan menggunakan proses hidrolisis asid, OPEFB akan di campurkan bersama asid sulfuric dan tindak balas yang berlaku akan menghasilkan, xylose. Produk yang diperolehi kemudiannya akan melalui proses penurasan untuk mengasingkan OPEFB yang tidak larut semasa eksperimen (Rahman et al., 2006). Dalam usaha untuk menghasilkan xylitol dari xylose, proses penapaian menggunakan mikroorganisma yang sesuai diperlukan. Oleh itu, xylose akan menjalani proses penapaian dengan menggunakan *Candida krusei* iaitu sejenis yis yang boleh menukar xylose untuk Xylitol selepas tempoh masa tertentu dengan budaya media yang sesuai. (Mohamad et al., 2009).

Terdapat tiga parameter yang digunakan dalam eksperimen iaitu kesan masa hidrolisis, kesan pH dan kesan jumlah hidrolisat terhadap penghasilan xylitol. Daripada keputusan yang diperolehi didapati bahawa tempoh masa untuk hidrolisis asid didapati 150 minit, pH adalah 2 dan nilai hidrolisat yang digunakan didapati 50 mL. Kesemua keadaan ini membawa kepada pengeluaran xylitol yang tinggi.

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LIST OF ABBREVIATIONS

g – Gram

g/L - Gram per litre

hr – Hour

L – Litre

M – Molar

mg – Miligram

mm – Minutes

ml – Mililitre

OFAT - One factor at time method

RSM - Response surface methodology

rpm - Round per minute

T – Temperature

U - Unit (enzyme activity)

°C- Degree Celsius

% - Percentage

1 INTRODUCTION

1.1 Motivation and statement of problem

Industries producing polyol sweeteners have registered a growing demand for the consumption of sugar-free and low heat value products. Among these, xylitol is an important sugar substitute with certain interesting physical and chemical properties which make it a high value compound for pharmaceutical, odontological and food industries (Swati et al., 2011). Xylitol basically consist of five carbon sugar alcohol which is found naturally in small amounts of fruits and vegetables (Mohamad et al., 2009) and has a lower caloric content (2.4 cal/g) than sucrose (4 cal/g) but with almost the same sweetness level (Juan et al., 2010). This compound is non and anticariogenic and its metabolism is insulin independent (Ricardo et al., 2007).

Xylitol can be produced from biomass, for example by using sugarcane bagasse (SCB) from the sugar-alcohol industry. The SCB excess is considered to be an environmental problem and its accumulation for long periods can even trigger spontaneous combustion. It is known that SCB is rich in polymeric compounds like cellulose, hemicelluloseand lignin. Almost one third of SCB is hemicellulose, an L-arabino-(4-O-methyl-D-glucurono)-D-xylan which is basically a xylose chain. Therefore, SCB can to be used to produce xylitol (Recardo et al., 2011). Besides that, other biomass that can be used to produced xylitol is Oil Palm Empty Fruit Bunch (OPEFB). This OPEFB fiber is a lignocellulosic waste which is renewable and contain high amount of xylan (approximately 24%), a polymer made of pentose sugar xylose (Abd Rahman *et al*., 2004). This make OPEFB one of the source of to produce xylitol. In past decades the palm oil residues became the most abundant biomass. This issue had lead to environmental problem such as attraction of pests and fouling. Loads of them was returned as compost and they are usually been burnt and this indirectly caused air pollution.

At present, large scale commercial production of xylitol is by an expensive catalytic hydrogenation of d-xylose from acid hydrolysis of lignocellulosics. Hence, it is

worthwhile to explore an alternative process for the effective production of xylitol using micro-organisms which make use of the semi synthetic media or detoxified hemicellulosic hydrolysate in order to reduce the manufacturing costs with minimal environmental and energy issues (Swati et al., 2011). Xylitol produced by biotechnological route also more economically viable because it requires milder conditions of pressure and temperature, and very little xylose purification since the enzymes or microorganisms specifically act on xylose-to-xylitol conversion (Ricardo et al., 2007).

In addition, xylitol has innumerous interesting properties for food, pharmaceutical and cosmetic products. The largest application of this compound is currently in oral products such as toothpaste, gum, mouthwash, nasal spray among others. Xylitol gives pleasant cool and fresh sensation due to its endothermic solution heat (34.8 cal g) which is ideal for gums. The sweetener power is similar to sucrose and amongst all the polyalcohols (sorbitol, arabitol and mannitol), it is the sweetest (Recardo et al., 2011).

1.2 Objective

The following are the objectives of this research:

- To determine the highest conversion of xylose to xylitol with different time of hydrolysis of oil palm empty fruit bunch (OPEFB) as parameter.
- To determine the most suitable pH for the production of xylitol.
- To determine effect of amount of hydrolysate to production of xylitol

1.3 Scope of research

The following are the scope of this research:

i) The scope of research for the production of xylitol will be covered from preparing the sample until producing final product.

ii) Acid hydrolysis effect, pH effect and amount of hydrolysate effect on the production of xylitol will be determined.

iii) Analysis of xylitol by using High Performance Liquid Chromatography (HPLC)

1.4 Main contribution of this work

The following are the contributions of this work:

1) The effect of all parameter can be used as reference in the future study for the production of xylitol.
2) The production of xylitol can be optimized based on this study.

1.5 Organisation of thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 provides a description of Oil Palm Empty Fruit Bunch (OPEFB) together with its application. General descriptions on the characteristics of the raw material, as well as the processes involved in the experiment are presented. This chapter also provides a discussion of the experimental techniques for analysis of the product (xylitol).

Chapter 3 gives a review of the method and the chemical used in the experiment. The work flow in achieving the final product are presented in details start from the preparation of the raw material, acid hydrolysis process, charcoal treatment process, sub-culture the yeast (*Candida Krusei*), fermentation process, cell disruption process and finally analysis of product using HPLC

Chapter 4 provide the result and the discussion from the experiment based on the three parameter been used which are the effect of hydrolysis time ,the effect of pH, and effect of amount of hydrolysate on xylitol production. Standard curve for xylitol together with final result on the xylitol production is presented based on the result from High Performance Liquid Chromatography (HPLC).

Chapter 5 draws together a summary of the thesis and outlines the future work which might be derived from result in this work.

2 LITERATURE REVIEW

2.1 Overview

This paper presents the experimental studies of production of Xylitol by *Candida Krusei* using Xylose from Oil Palm Empty Fruit Bunch as substrate. The studies involved the determination on effect of hydrolysis time and also the effect of pH on production of Xylitol. The analysis of the product was done by using High Performance Liquid Chromatography (Supercosil LC NH_2 column with R.I detector) and the standard curve was plotted in order to determined the concentration for each sample.

2.2 Introduction

Elaeis guineensis Jacq which is commonly known as the oil palm is the most important species in the genus Elaeis which belongs to the family Palmae. The second species is *Elaeis oleifera* (H.B.K) Cortes which is found in South and Central America and is known as the American oil palm. Although significantly lower in oil-to-bunch content than its Africa counterpart, E. oleifera has a higher level of unsaturated fatty acids and has been used for production of interspecfic hybrids with *E. guineensis*. The oil palm is an erect monoecious plant that produces separate male and female inflorescences (Teoh, 2002). The oil palm tree parts have many uses. For instance the oil palm fruit can be process to produce cooking oil, oil palm trunk used for manufacturing plywood while oil palm trunk is used as a roughage source or as a component in compound feed for ruminants. The oil palm tree can be shown as in Figure 2.1:

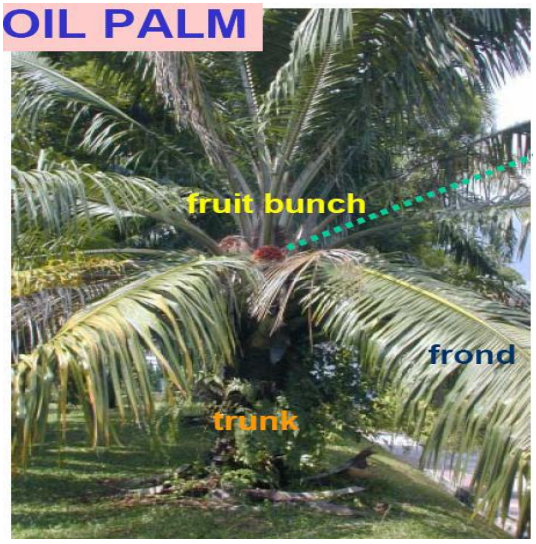

Figure 2.1 Oil Palm Tree

Harvesting process oil palm fruit commences about 24 to 30 months after planting and each palm can produce between eight to 15 fresh fruit bunches (FFB) per year weighing about 15 to 25 kg each, depending on the planting material and age of the palm. Each FFB contains about 1000 to 1300 fruitlets; each fruitlet consists of a fibrous meoscarp layer, the endocarp (shell) which contains the kernel (Teoh, 2002). Present day planting materials are capable of producing 39 tonnes of FFB per ha and 8.6 tonnes of palm oil and actual yields from good commercial plantings are about 30 tonnes FFB per ha with 5.0 to 6.0 tonnes oil (Henson. 1990).

OPEFB fiber is a lignocellulosic biomass which is renewable and contain high amount of xylan (approximately 24%), a polymer made of pentose sugar xylose and hydrolyzing acid such as sulfuric acid, hydrochloric acid or hydrofluoric acid can be used as catalyst for reduction of xylan polymer to monomeric sugar xylose (Abd Rahman *et al*., 2004). The oil palm empty fruit bunch (OPEFB), obtained after stripping the fruit from the bunch, and it is generated by the palm oil industry is about 7.3 $x10^6$ tonnes annually (Chua 1991). This biomass has many useful applications in industry and the picture of OPEFB show as Figure 2.2:

Figure 2.2 Oil Palm Empty Fruit Bunch

This large volume of agricultural waste has not been effectively utilized for its lignocellulosic materials and appears to be a viable alternative as a cheap source of substrate for cellulase production (Sreekala *et al.,* 1997).

Lignocellulosic biomass refers to plant biomass that is composed of cellulose, hemicellulose, and lignin. Lignocellulosic biomass is also a potential source of starting materials for many industrial processes. The advantage of this biomaterial is that its processing is or will shortly become less expensive (Lucian, 2008). Besides that, lignocellulosic biomass has long been recognized as a potential low cost source of mixed sugars for fermentation. Cell walls in lignocellulosic biomass can be converted to mixed-sugar solutions plus lignin-rich solid residues by sequential use of a range of thermochemical pretreatments and enzymatic saccharication (Brown, 2003). The amount of the component in lignocellulosic can be summarized as in table 2.1:

Table 2.1: General composition of lignocellulosic biomass

General composition of lignocellulosic biomass	Composition percentage
Cellulose	30-50%
Hemicellulose	20-40%
Lignin	15-25%
Other	5-35%

Cellulose or ß-1-4-glucan is a polymer of glucose made of cellobiose units with about 2,000 to 27,000 glucose residues. These chains are packed with hydrogen bonds in so called 'elementary fibrils' originally considered to be 3 to 4 nm wide and contain about 36 chains. These elementary fibrils are then packed in so called microfibrils, where the elemnatry fibrils are attached to each other by hemicellulose as well as other polymers such as pectin and covered by lignin (Mohammad and Keikhosro., 2007).

The basic structural component of plant cell walls, cellulose comprises about 33 percent of all vegetable matter (90 percent of cotton and 50 percent of wood are cellulose) and is the most abundant of all naturally occurring organic compounds. The acetal linkage is beta which makes cellulose different from starch (Retrieved from http://www.britannica.com/EBchecked/topic/101633/cellulose). The structure of cellulose in Figure 2.3.

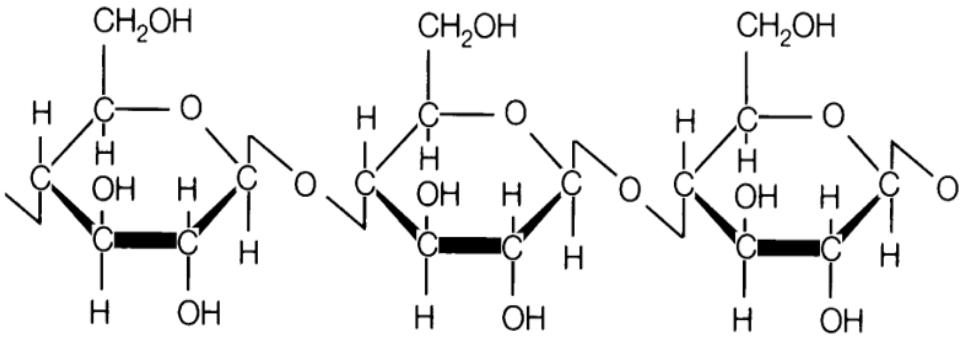

Figure 2.3 Structure of cellulose

Hemicellulose is a high polymeric carbohydrate in the cell walls. The three components of lignin, hemicellulose and cellulose are tightly bound to each other in the biomass. Hemicellulose acts as a bonding agent between cellulose and lignin. In order to convert these biomass to desired product, the biomass has to be broken up into the individual components first before the molecular chains within each component can be broken up further into simpler molecules. Hemicellulose can be broken up or hydrolysed to yield simpler molecules such as arabinose, mannose, glucose, galactose, xylose (the most abundant) and uronic acid, while cellulose can

be hydrolysed to yield glucose molecules. Compared to hemicellulose and cellulose, lignin on the other hand cannot be easily hydrolysed. While hemicellulose can easily be hydrolysed with boiling dilute acid, the same cannot be said of cellulose.(Lim, 2004)

Lignin is found in all vascular plants, mostly between the cells, but also within the cells, and in the cell walls. In nature it is very resistant to degradation, being held together with strong chemical bonds; it also appears to have a lot of internal H^+ bonds. It is bonded in complex and various ways to carbohydrates (hemicelluloses) in wood. Lignin is actually not one compound but many. All are complex, amorphous, three-dimensional polymers that have in common a phenylpropane structure, that is, a benzene ring with a tail of three carbons. In their natural unprocessed form, they are so complex that none of them has ever been completely described, and they have molecular weights that my reach 15,000 or more (Ellen, 1991). The structure of lignin is illustrated as in Figure 2.4.

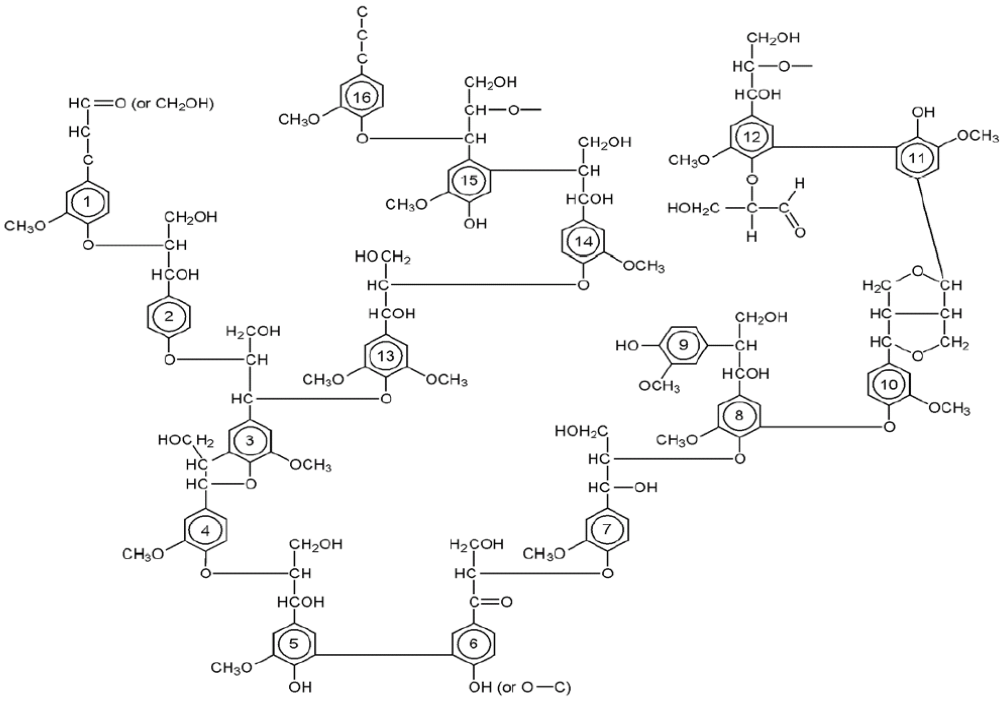

Figure 2.4 Structure of lignin

2.3 Previous work on xylitol

There are few methods in order to produce xylitol. Firstly by using microbiological fermentation method. As the hemicellulosic fraction contains amounts of polymers of other sugars, the chemical process includes expensive separation and purification steps to remove these by-products from xylose or xylitol cab be produced with natural xylose-utilizing yeasts, especially species of the genus Candida, such as C. blankii , C. boidinii , C. Guilfiermondii, C. Petiicufosa,C. shehatae , C. Tropicalis, and C. Utilis. However the use of yeasts, has the disadvantage of low xylitol productivity. In xylose metabolism to produce xylitol, yeasts use two-step oxido-reduction reactions to convert xylose to xylitol and then to xylulose. The first reaction is the reduction of xylose to xylitol with a NADPH- or NADH-dependent xylose reductase. Xylitol is either excreted from the cell or oxidized to xylulose by the second reaction with a NAD+-dependent xylitol de- hydrogenase (Sang Yong et al., 1997). Research investigations on dilute acid hydrolysis of various raw materials such as sugar cane bagasse, sorghum straw, corn cobs and eucalyptus wood have been carried out by several workers. From the research studies it was revealed that under controlled treatment conditions, acid hydrolysis of lignocellulosic biomass mainly produced xylose from xylan with the cellulosic and lignin fractions remaining unaltered (Mohamad *et al.*, 2009).

The other way to produce Xylitol is by enzymatic process which is the new biotechnological alternative forthis microbiological process which can achieve 100% of conversion. This high conversion value is due to the direct transformation of xylose into xylitol which cannot be achieved by the fermentative process because of deviation of xylose to cell maintenance. The enzymatic process consists the direct reduction of xylose to xylitol by the enzyme xylose reductase assisted by the coenzyme nicotinamide adenine dinucleotide phosphate in its reduced form(NADPH). Further, the NADP can be reduced again ina coupling enzymatic reaction to make the process more economically attractive. This work deals with the glucose dehydrogenase system in which glucose is oxidized to gluconic acid (gluconate) mediated by glucose dehydrogenase and NADP is reduced to NADPH. A simple scheme of xylitol enzymatic reaction is shown in Figure 2.5:

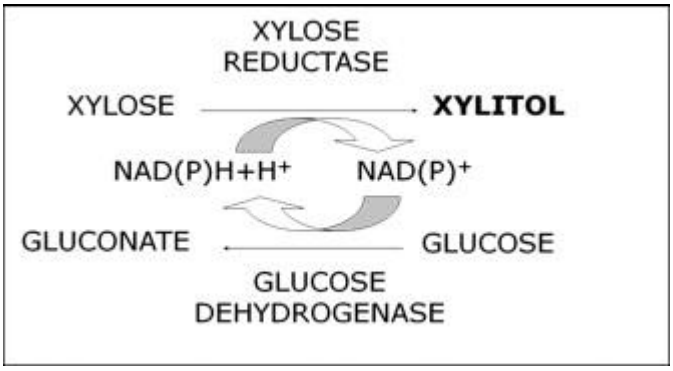

Figure 2.5 Production of xylitol (using enzyme)

Other method that can be used to produced Xylitol is by chemical synthesis through catalytic hydrogenation of D-xylose. However, this process is expensive given that D-xylose must be purified prior to the synthetic reaction (Azuma et al., 2000).

Pretreatment involves the alteration of biomass so that hydrolysis of cellulose and hemicellulose can be achieved more rapidly and with greater yields. Possible goals include the removal of lignin and disruption of the crystalline structure of cellulose (Harmsen *et al.*, 2010). Figure 2.6 illustrate the effect of pretreatment on lignocellulosic biomass.

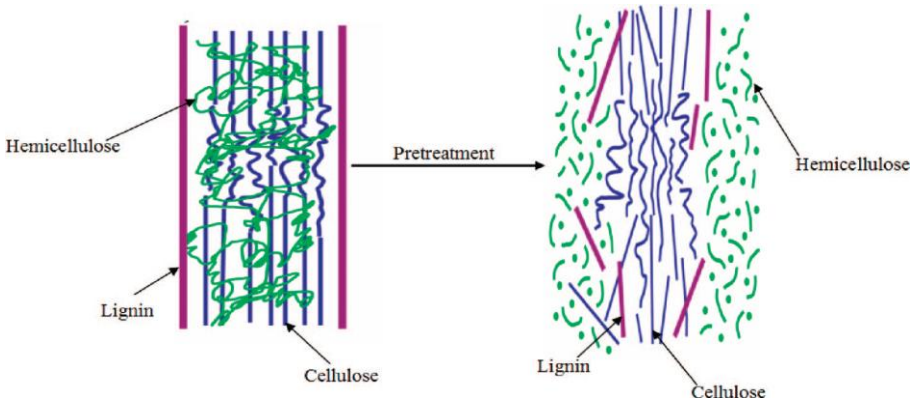

Figure 2.6 Schematic presentation of effects of pretreatment on lignocellulosic biomass

Acid hydrolysis is one of method of pretreatment ptocess to produce xylose. This process is done by contacting the empty fruit bunch fibre with Sulphuric Acid at certain concentration. The lignocellulose size is firstly, reduced to a very small fine particle by using a grinding mill that may increase the surface area and reduce the diffusion problem related to the reactant involved (Najafpour *et al*., 2007). Hydrolysis reaction is generally controlled by hydrogen ion concentration (H^+). At the same time, the cellulose fragment dissolution performed as a function of hydroxyl ion concentration (Ladisch, 1989). It is required to alter the structure of cellulosic biomass to make cellulose more acces-sible to the enzymes that convert the carbohydrate polymers into fermentable sugars.

Xylan is more susceptible to hydrolysis by mild acid treatment due its amorphous structure compared to cellulose which needs severe treatment conditions for its crystalline nature (Mohamad *et al.*, 2009). Although xylose was the main sugar obtained from hemicellulose, other by products such as glucose, acetic acid, furfural etc., will also be produced in low amounts during the hydrolysis process. It was also reported that amount of sugar released during hydrolysis, depended on type of raw material and operating conditions of the experiment (Rahman et al., 2006).

The acidic pre-treatment of lignocellulosics hydrolyzes the hemicellulose fraction, enabling subsequent enzymatic digestion of the cellulose in fermentation reaction. However, the non-specificity of acidic treatment led to the formation of complex sugars and compounds inhibitory to the microorganisms for ethanol production. These inhibitors can be divided into three major groups which are organic acids (acetic, formic and levulinic acids), furan derivatives (furfural and 5-hydroxymethylfurfural), and phenolic compounds affecting overall cell physiology and often result in decreased viability, and productivity. The detoxification process will overcome the fermentation inhibitors of lignocellulosics. The detoxification of hemicellulose hydrolysates, by activated charcoal is known to be a cost effective with high capacity to absorb compounds without affecting levels of sugar in hydrolysate. The effectiveness of activated charcoal treatment depends on different process variables such as pH, contact time, temperature and the ratio of activated charcoal taken versus the liquid hydrolysate volume (Anuj et al., 2010).

Yeasts are unicellular fungi that are found commonly in natural environments. There are about 1500 species currently known to science and it is estimated that less than 1% of all species have been described. A relatively unique trait of yeasts that distinguishes them from most other life forms is that they are capable of both aerobic and anaerobic metabolism. This quirk turns out to have an important impact on brewing, as brewers need to leverage both forms of metabolism in order to achieve a high-quality finished product. One example of yeast is *Candida Krusei* which can be used in fermentation process. According to Samaranayake *et al.* (1994) said that in contrast to a majority of other *Candida sp.* which are ovoid in shape, the cells of *Candida krusei* are generally elongated and have the appearances of "long grain rice". A feature which the *Candida krusei* measures 2.2 -5.6 x 4.3 -15.2 µm with wide variation in the length and breadth of the isolates. The macrostructure of *Candida krusei* shows in Figure 2.7

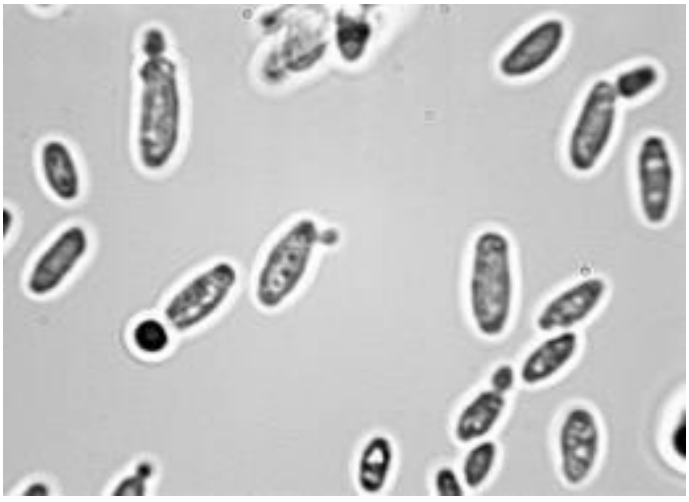

Figure 2.7 Structure of *Candida krusei*

The production of xylitol from the past research was based on certain condition in order to get high production of xylitol. From the previous study, hydrolysis of oil palm empty fruit bunch fiber was performed at operating temperature 120°C using various concentration of sulfuric acid (2–6%) and reaction time (0–90 min). However, according on optimization studies on acid hydrolysis of oil palm empty

fruit bunch, the optimum reaction temperature, reaction time and acid concentration found were 119°C, 60 min and 2%, respectively (Rahman et al., 2006). Acid hydrolysis of OPEFB biomass was carried out in 125 ml Erlenmeyer flasks. The media consisted of 2–6 g H_2SO4/100 g liquor using a charge of 1 g OPEFB fiber/ 8 g liquor on dry basis. Operating temperature of hydrolysis was varied between 100°C and 130°C and samples were collected at various time intervals in the range of 30–90 min. It was found that under optimum conditions, xylose yield and selectivity were 91.27% and 17.97 (g/g), respectively when operating temperature, reaction time and acid concentration were 119 C, 60 min and 2%, respectively. Thus it is to be mentioned that under controlled treatment conditions, oil palm empty fruit bunch fiber waste can be fruitfully utilized as a potential source of xylose, which can be a starting raw material for production of various chemicals, especially xylitol by microbial conversion process (Rahman et al., 2007).

According to Journal of Applied Science reported by Mohamad *et al.*, (2009), yeast grows well in acidic condition with pH between 3.5 and 4.0 and the optimum pH for Xylitol production appears to be 5.5 for *D. Hansenii*, and in the range of 4-6 for *Candida sp*. The experiment was done by taking pH as the parameter and it was found that drastic increase of xylitol production after 54 hour of fermentation when the pH was increase from 2 to 4. However, in the other past study, the most suitable pH value for the production of Xylitol was found to be from the range of 4-6 whereby more conversion of Xylitol produced between this range of pH value (Fabio *et al*., 2006)

2.4 Analysis methods for Xylitol

The fermentation process of the hydrolysate with Candida Krusei will produce Xylitol and the detection of Xylitol produced can be analyzed by High Performance Liquid Chromatograph (HPLC) using SUPELCOSIL LC-NH2 column and RI detector. The aqueous acetonitrile (75%) was used as mobile phase with flow rate of 1.5 ml min−1 and oven temperature was maintained at 50°C. As the experiment will be done by using HPLC, ultra pure water is used for another (25%) of mobile phase.

2.5 Summary

OPEFB has many benefits to human and also environment. Rather than waste the biomass and burn it for nothing beside pollute the environment, OPEFB actually have high commercial value. Practically from OPEFB, xylitol can be produced, and this desired product has wide application in industries. Xylitol has received global demand mainly due to its insulin-independent metabolism, anticariogenecity, high sweetening power, and pharmacological properties. Xylitol is currently approved for usage in foods, pharmaceuticals, and oral health products in more than 50 countries (Sukiran *et al.,* 2009). By using fermentation process, enzymatic method it is expected to make a substantial increase in productivity of xylitol. From past research (Ikeuchi *et al.,* 1999) *Candida* sp. was characterized as a good potential candidate for xylitol production from xylose. Compared to the chemical process for xylitol production, the fermentative potential using microorganisms allows the development of a biological process. Finally, this paper present the Xylitol production study based on the parameter of time of hydrolysis and pH and the result will be compare with the past study which show that the optimum time for the production of xylitol was 60 minutes of hydrolysis time and the pH in the range of 4-6.

3 MATERIALS AND METHODS

3.1 Overview

This paper present a methodology of producing Xylitol by using Candida Krusei using xylose from Oil Palm Empty Fruit Bunch (OPEFB) as substrate. The process described starting from preparing the raw material until the fermentation process and finally the analysis of product by using High Performance Liquid Chromatography (HPLC). The operating condition for each step in the process will be explained in details and the chemical and equipment used in the experiment also will be presented in this chapter.

Overview of Process Methodology

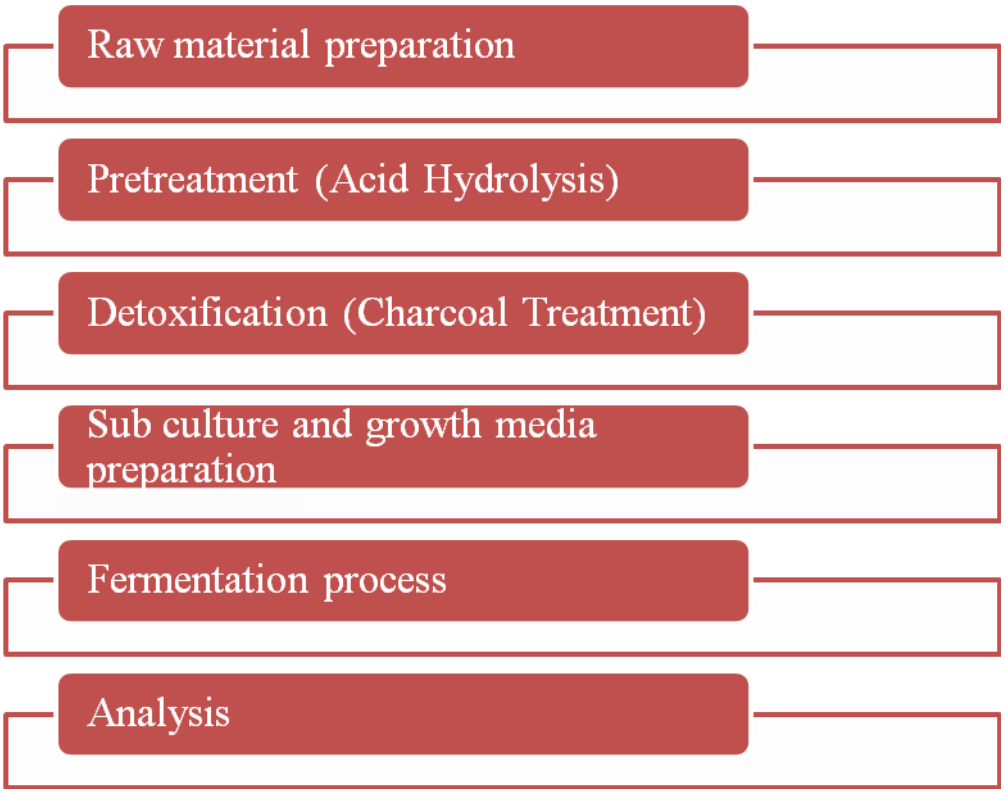

Figure 3.1 Summarize of Methodology

3.2 Introduction

Xylitol is the main composition that needs to be produced in this experiment. The concentration of the Xylitol produced will show the optimum value of the parameter been used in the experiment. *Candida Krusei* will react with the hydrolysate and converting xylose in the hydrolysate into Xylitol.

3.3 Chemicals and equipment

The chemicals and equipment used in this study is of analytical grades, and they are summarizes in Table 3.1 and Table 3.2.

Table 3.1 List of equipment used

Equipment	Brand	Principal Used
Rotary shaker	Infors Ht	Fermentation
Centrifuge	Kubota Corporations	Fermentation
Refrigerated centrifuge	Eppendorf	Fermentation
Shaking water bath	BS-21	Analysis
Oil Bath	Memmert	Analysis
pH meter	Mettler Toledo	Analysis
HPLC	Agilent Technologies	Analysis
Homogenizer	D.I Scientific	Analysis

Table 3.2 List of chemical used

Chemical	Supplier	Principal used
Xylose	Sigma, Aldrich	Fermentation
Xylitol	Sigma, Aldrich	Analysis
Yeast extract	Sigma, Aldrich	Fermentation
KH_2PO_4(monosodium phosphate)	Sigma, Aldrich	Fermentation
$Mg.SO_4.7H_2O$ (magnesium sulphate heptahydrate)	Sigma, Aldrich	Fermentation
Peptone	Fluka	Fermentation
Sodium Hydroxide(NaOH)	Sigma, Aldrich	Analysis
Acetonitrile	Sigma, Aldrich	Analysis
Sulphuric Acid	Sigma, Aldrich	Analysis

3.4 Methodology

Oil palm empty fruit bunch (OPEFB) fiber will be collected from local palm oil mill. Then, the next step will be a sun dried process (Rahman *et al*., 2006). However in order to make the OPEFB dry faster, OPEFB need to be tear or shred to provide higher surface area for sun dried drying process. OPEFB then will be sun dried for 3 hours and the dried OPEFB will be grinded to particle size less than 1 mm using grinder. The homogenized OPEFB biomass will then oven-dried at 105°C for overnight (Rahman *et al*., 2006). Figure 3.2 and Figure 3.3 shows the picture of grinding machine and OPEFB that had been grinded.

Figure 3.2 Grinding machine

Figure 3.3 Grinded Oil Palm Empty Fruit Bunch

The acid hydrolysis of OPEFB biomass will be carried out in 125 ml Erlenmeyer flasks and the acid that will be used is Sulphuric Acid. The media consist of 2–6 g H_2SO_4/100 g liquor using a charge of 1 g OPEFB fiber/ 8 g liquor on dry basis (Rahman *et al*., 2006). The operating temperature that will be used for this acid hydrolysis is 115°C for time period of 60 min with acid concentration 2%. After reaction completed, the solids (insoluble OPEFB) will be removed from aqueous

solution by filtration. Figure 3.4 show the image of hydrolysate after hydrolysis with sulphuric acid.

Figure 3.4 Hydrolysate

Charcoal detoxification of hydrolysate will be carried out by contacting the hydrolysate and activated powdered charcoal using the mass ratio $10gg^{-1}$ at room temperature and stir the mixture in an orbital shaker for one hour at 200 rpm. The liquid phase will be centrifuged with 3500 rpm and the hydrolysate was recovered by filtration (Abd Rahman *et al*., 2004). Figure 3.5 illustrate the hydrolysate after centrifugation proce

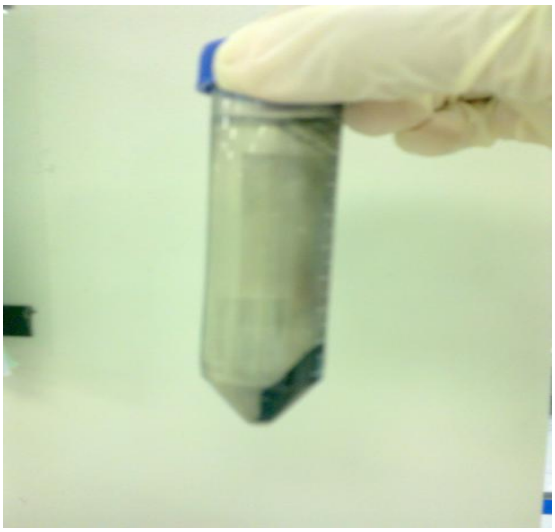

Figure 3.5 Hydrolysate after centrifuged

In this experiment, the bioconversion of xylose to xylitol will be performed by yeast, *Candida Krusei*. In order to grow this yeast, pre-culture medium must be made. *Candida Krusei* was aseptically transferred onto a plate containing sterile yeast extract agar (YEA). The strain was subculture once by previous study and incubated at 30 °C for 3 days or 24 hours as illustrated as in Figure 3.6. Then, the stock cultures were sealed and stored in refrigerator at 4 °C until further required usage.

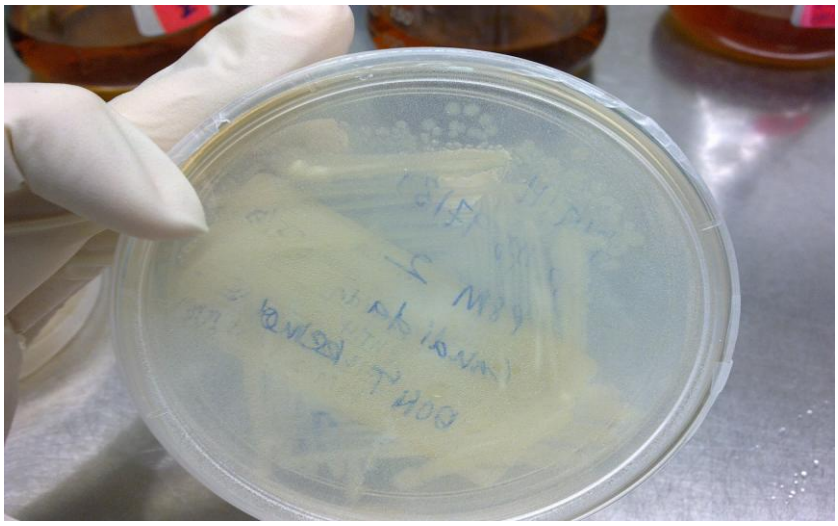

Figure 3.6 Agar plate straining of *Candida krusei*

Inocolum medium was prepared whereby the inocolum medium consist of 3 days old strains were then transferred into inoculum medium. The inoculum medium consists 30g/L of xylose, 5g/L peptone and 3 g/L yeast extract. Five 250 mL shake flask were filled with the volume of 100 mL of inocolumn medium and the *Candida krusei* on the plate was streak with sterilize inocolumn loop and was put into the inocolum media. Then, the flask was incubated for 24 h at 30°C on rotary shaker at 200 rpm. Figure 3.7 show Inoculum of *Candida krusei.*

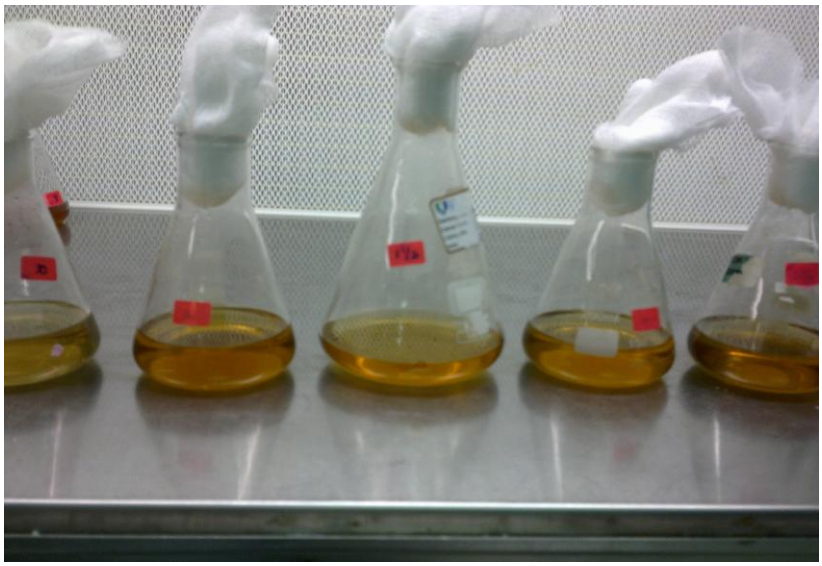

Figure 3.7 Inoculum of *Candida krusei*

10% of the inocolum volume was transferred into 250 mL shake flask after 24 hours of incubation. The fermentation medium consists of (L^{-1}) 20 g xylose, 1 g peptone, 4 g yeast extract, 0.5 g $MgSO_4.7H_2O$ and 2% KH_2PO_4. 50 mL amount of hydrolysate that had been trough hydrolysis process at different time (30 minutes, 60 minutes,90 minutes,120 minutes and 150 minutes) and 50 mL fermentation medium were mixed up together in five different shake flask. The hydrolysate was autoclaved before been put into the fermentation medium. The cultures were incubated for 48 hours at 30°C by using incubator shaker at the speed of 220 rpm. Figure 3.8 show the fermentation medium.

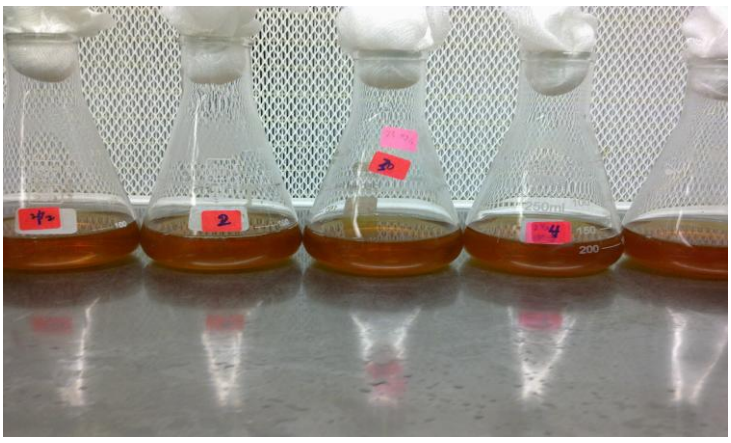

Figure 3.8 Fermentation of *Candida krusei* in 250 mL of shake flask

3.5 Data Analysis

After the fermentation process ended, the culture brooth was centrifuged by refrigerated centrifuge for 15 minutes at 10 000 rpm and at 4°C. The biomass from the centrifugation was then been homogenized by using hand held rotor stator homogenizer for 20 minutes for each sample in order to disrupt the cells. The disrupted cell then been added with 45 ml distilled water and again be centrifuged at the same condition as before. The supernatant was taken for the analysis of Xylitol while the biomass was filtered and the dry weight of the biomass was calculated. Figure 3.9 show the picture of centrifuged sample while Figure 3.10 show the picture of samples been homogenized.

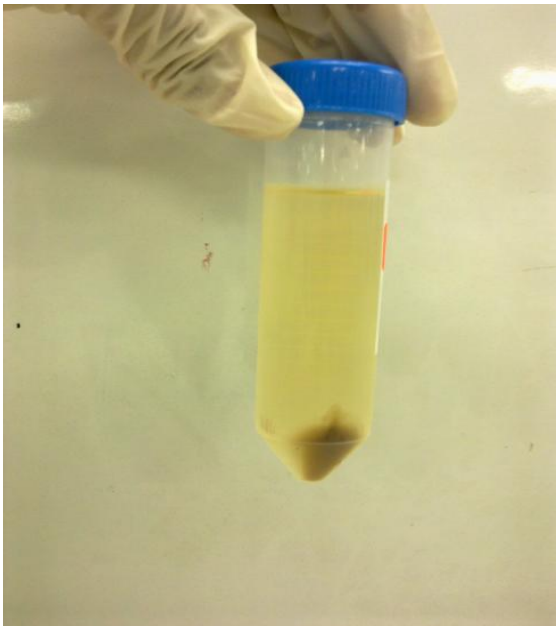

Figure 3.9 Centrifuged sample

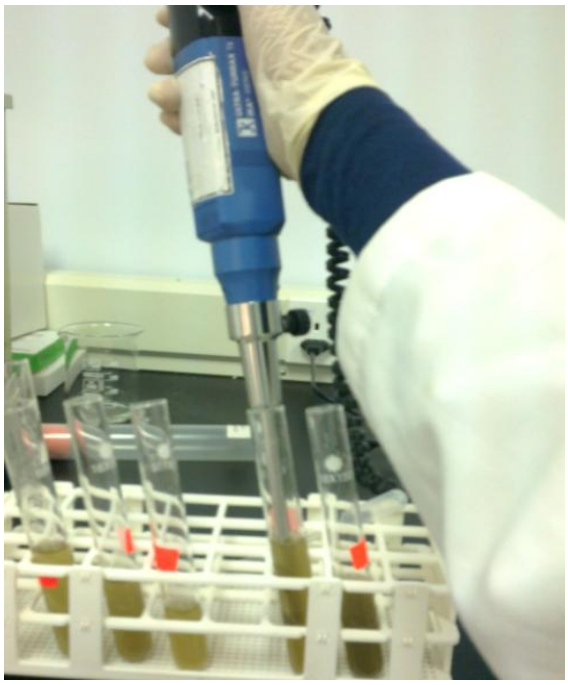

Figure 3.10 Homogenized samples

The samples were transferred into vial tube before undergo the analysis process while the mobile phase that been used must be filtered using vacuum filter and undergo de-gassed process in order to remove bubble that might interrupt the analysis process. The samples then were analyzed for xylose and xylitol by using high performance liquid chromatography (HPLC) using Supelcosil LC-NH_2 column and RI detector. Aqueous acetonitrile will be used as mobile phase with flow rate of 1.5 ml/min and oven temperature maintain at 50°C. Figure 3.11 show vacuum filter that been used.

Figure 3.11 Vacuum filter

Figure 3.12 show the sample been transferred into the vial tube.

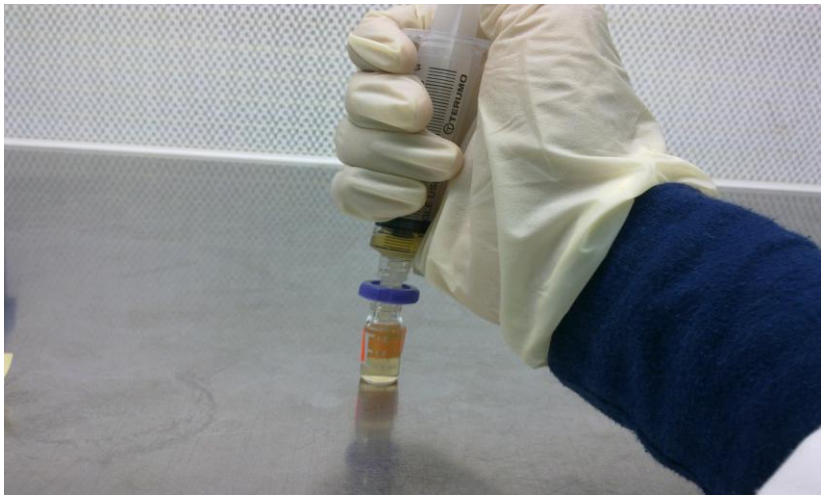

Figure 3.12 Sample in vial tube

Figure 3.13 show High Performance Liquid Chormatography (HPLC) that been used for analysis pupose..

Figure 3.13 High Performance Liquid Chormatography

3.6 Summary

The experiment was run by using all the method that been described. The standard curve was plotted and the concentration of each sample was found. The experiment was then repeated for the next parameter which was pH and the amount of hydrolysate. The pH was controlled by using 1 M Sodium Hydroxide. Five pH value were set which are 1,2,3,4,5 and the sample concentration again been determined. Figure 3.14 show pH meter been used to check pH value.

Figure 3.14 pH meter

4 RESULT AND DISCUSSION

4.1 Overview

This chapter discuss on the result obtained from the experiment to produce Xylitol by according to the parameter which are effect of time of hydrolysis, pH and the amount of hydrolysate used in the fermentation process. Candida krusei was used in the fermentation process which conducted under the operating temperature of 30°C for 48 hours in incubator shaker at 220 rpm. The samples obtained after the fermentation then was analyzed using High Performance Liquid Chromatography (HPLC, Agilent Technologies) to get the concentration of Xylitol produced.

4.2 Introduction

Xylitol calibration curve was plotted and was used to determine the concentration of xylitol in an unknown sample by comparing the unknown to a set of standard samples of known concentration. By using HPLC, the absorbance values obtained were plotted against the concentration of xylitol. The following Equation (4.1) was obtained.

$$y = 0.0231x \qquad (4.1)$$

Whereby, y is equal to the area value while x is equal to the concentration of xylitol (g/L). Figure 4.1 shows the calibration curve of of xylitol for this experiment. The graph was plotted with area versus concentration of xylitol. The graph exhibited that the area increased when the xylitol concentration (g/L) increased.

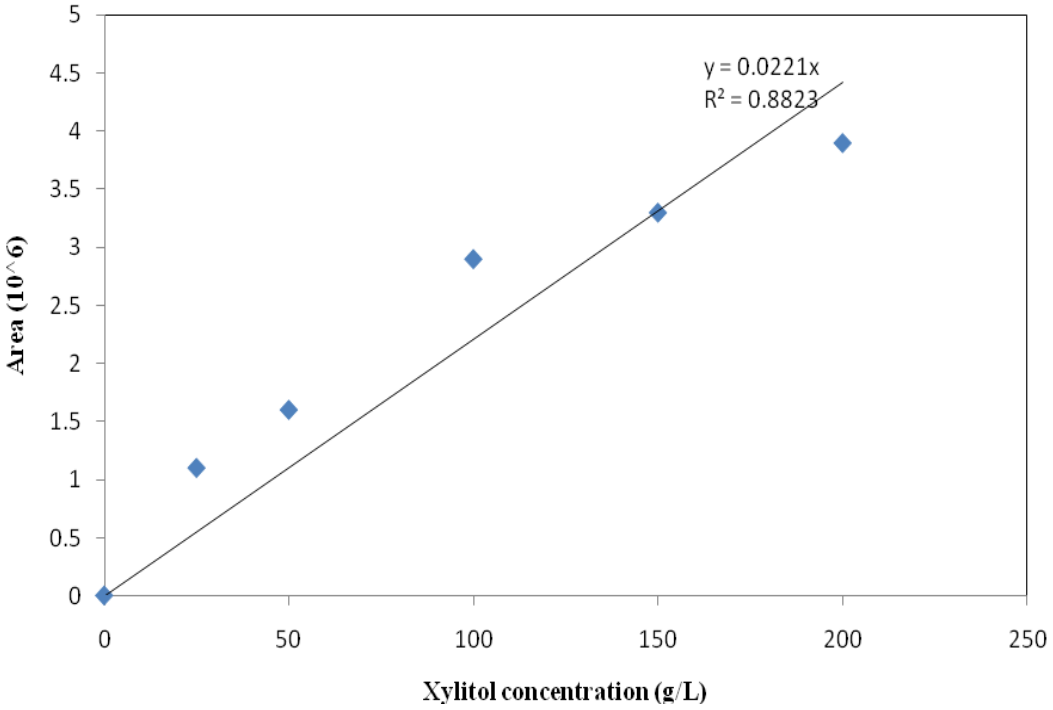

Figure 4.1 The calibration curve of xylitol

4.3 Effect of hydrolysis time to the production of xylitol

The effect of hydrolysis time using sulphuric acid was done in order to the determined the amount of xylitol can be produced based on the time of hydrolysis of Oil Palm Empty Fruit Bunch (OPEFB). Hydrolysis process was a pre-treatment process in order to produce xylose before the xylose was used as compound to produce xylitol. The amount of OPEFB to amount of acid sulphuric acid been used ratio was 1:8 for all sample. All samples was run in the same operating condition but at different time of hydrolysis which are 30 minutes, 60 minutes, 90 minutes, 120 minutes, and 150 minutes. Figure 4.2 illustrate the effect of hydrolysis time (minutes) to the production of xylitol.

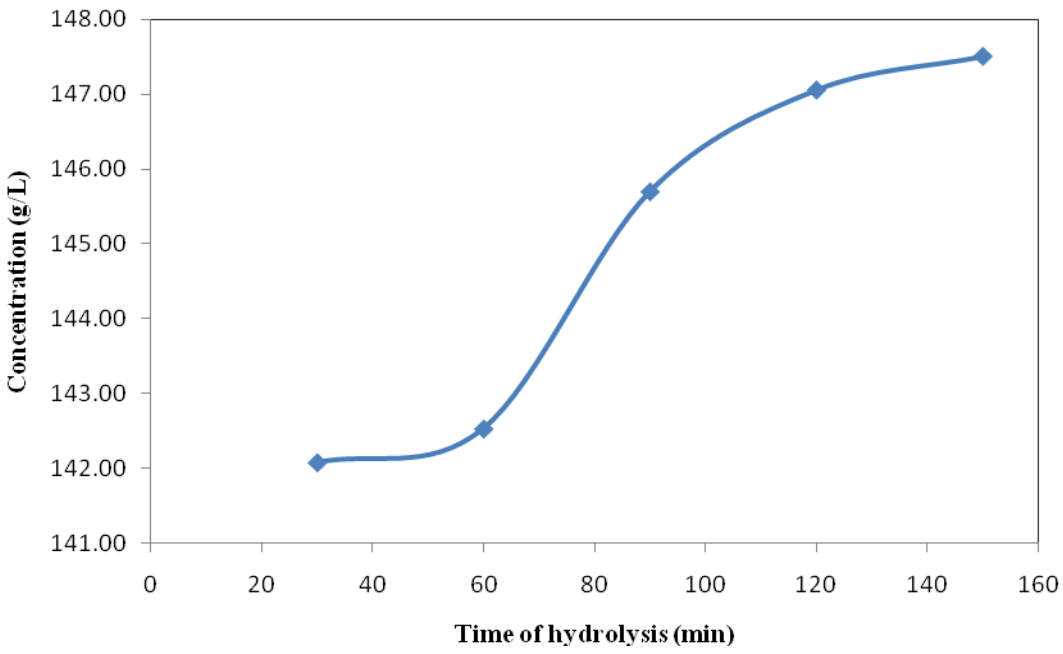

Figure 4.2 Effect of time of hydrolysis to production of xylitol

Based from Figure 4.2, it was found that at 30 minutes, the amount of xylitol produced was the lowest with the concentration of xylitol was 142.08 g/L while during 150 minutes, the production of xylitol was the highest with the concentration of xylitol was 147.51 g/L. It shows that the longer time of hydrolysis yield the higher amount of xylitol production. This was due to the effect of hydrolysis process which mixture of monosaccharides was produced during the process including xylose. The longer time of hydrolysis give better effect for the reaction between the substrate (OPEFB) with the diluted acid sulphuric solution which yields to higher production of xylitol while shorter time for hydrolysis process give less time for reaction of the acid sulphuric dilution with substrate (OPEFB) which lead to low production of xylitol. This phenomenon was similarly as reported by P.Jeevan *et al.*, (2011) whereby the total concentration of hemicellulose carbohydrates increased when longer reaction times were used, with the higher xylitol concentration being obtained.

4.4 Effect of pH on production of xylitol

The next parameter that been used in the experiment was pH. The effect of pH on the production of xylitol was studied in order to obtain the optimum pH for the production of xylitol. The pH was manipulated with the range of pH from 1 until 5 and the pH was controlled by using Sodium Hydroxide (NaOH) 1 M solution. The hydrolysate used in the fermentation process was the hydrolysate that had been through hydrolysis process for 150 minutes since 150 minutes show the highest xylitol production previously. Figure 4.3 shows the effect of pH to production of xylitol.

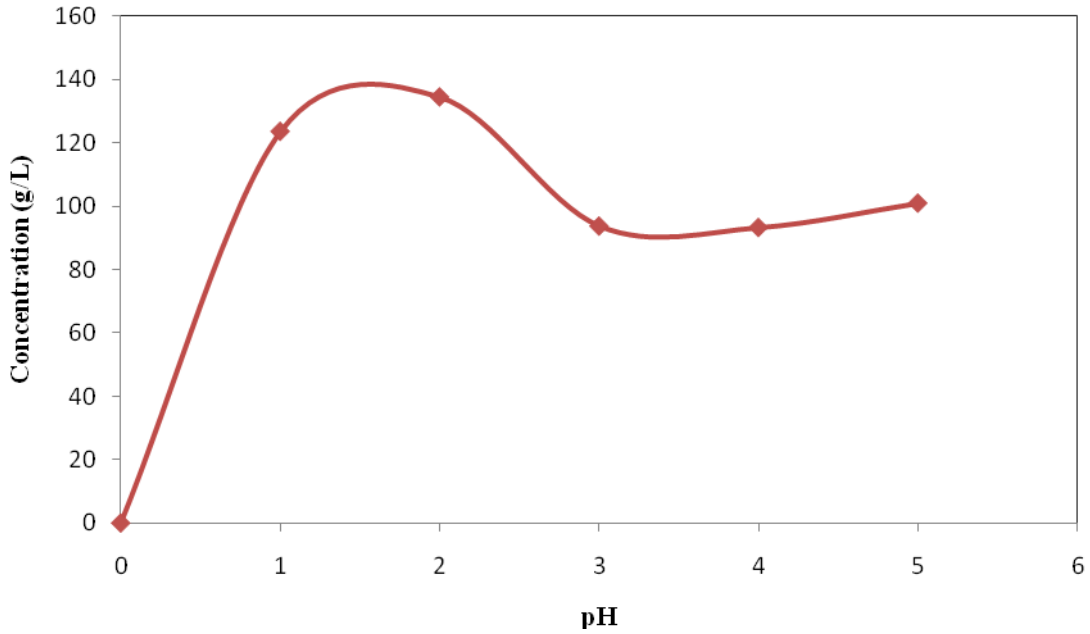

Figure 4.3 Effect of pH on production of xylitol

From figure 4.3, it was found that pH that produced highest xylitol amount was pH 2 with the concentration of 134.39 g/L while at pH 4, the production of xylitol was the lowest with the concentration of xylitol 93.21 g/L. In the other past study, the most suitable pH value for the production of xylitol was found to be from the range of 4-6 by using yeast whereby more conversion of xylitol produced between this ranges of pH value. However, it was also found in the past research that yeasts usually grow well in acidic medium with pH between 3.5 and 4.0, but the tolerance threshold was in the wide range which was between the range of 2.5–8.0 and optimum pH for

xylitol production appears to be strongly species-dependent, being 5.5 for *D. Hansenii*, and 2.5 for *Candida tropicalis* (Fabio *et al.*, 2006). As for the result obtained in this experiment, pH 2 was found to be the optimum pH for *Candida Krusei* species and it was in the range of the pH tolerance threshold.

4.5 Effect of amount of hydrolysate to production of xylitol

The last parameter in the experiment was the effect of amount of hydrolysate to the production of xylitol. This parameter was study in order to obtain the optimum hydrolysate amount to achieve high production of xylitol. The experiment was performed with five different amount of hydrolysate which are 10 mL, 20 mL, 30 mL, 40 mL and 50 mL that been mixed together with the fermentation medium. After the fermentation process, the result was obtained as in Figure 4.4.

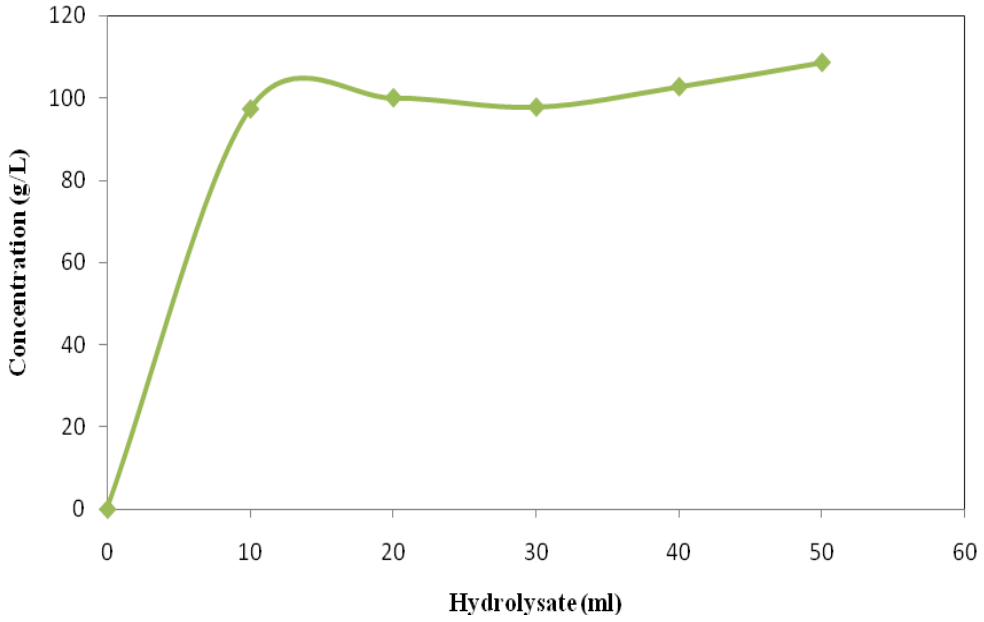

Figure 4.4 Effect of the amount of hydrolysate (mL) to the production of xylitol

From Figure 4.4, it was found that the more hydrolysate (mL) was introduced into the fermentation medium, the higher the production of xylitol. The highest xylitol produced was when the hydrolysate was 50 mL whereby the concentration of xylitol was 108.59 g/L while the lowest xylitol produced was when the hydrolysate amount was 10 mL with the concentration of xylitol 97.28 g/L. Hydrolysate was the source of xylose that been converted into xylitol by *Candida Krusei*. Higher amount of hydrolysate provided higher amount of xylose and indirectly increase the amount of xyitol produced during fermentation. However, it was found that the concentration of the xylitol produced decreased when the amount of hydrolysate was 30 mL whereby the concentration of xylitol was found to be 97.74 g/L. The amount of xylitol produced at 20 mL was 100.00 g/L and this show there was some decreased of the amount of xylitol produced. This occur due to the inhibitor that produced during the hydrolysis process previously that effecting the amount of xylose produced and this phenomenon also might be happened due to the amount of oxygen dissolved in the fermentation brooth. Oxygen was required for the efficient uptake of xylose. Under low dissolved oxygen (DO) conditions, the electron transport system was not able to oxidize NADH efficiently, this causes the imbalance of NADH that leads to the accumulation of xylitol. An increase in DO will enhance cell growth and xylose fermentation (Wiley J.,1999).

4.6 Summary

There were three parameters in this study. All the parameter have same objective which was to determine the most effective operating condition to produce xylitol. The best time for hydrolysis was found to be 150 minutes, the pH was 2 and the value of the hydrolysate was found to be 50 mL.

5 CONCLUSION

5.1 Conclusion

The main objective of this research is to study the effect of time of hydrolysis, pH and amount of hydrolysate (%) on xylitol produced from *Candida krusei* using xylose from Oil Palm Empty Fruit Bunch (OPEFB) as a substrate was achieved. From the result obtained, it can be concluded that the most suitable time for acid hydrolysis for higher production of xylitol was at 150 minutes where the concentration of xylitol yield was 147.51 g/L. For the pH effect on the production of xylitol, it was found the pH 2 was the best pH with the concentration xylitol produced was 134.39 g/L. Finally, the last parameter which was the amount of hydrolysate, show that the higher amount of hydrolysate produced more xylitol with the concentration of 108.59 g/L for 50 mL hydrolysate in the fermentation medium.

5.2 Future work

Several numbers of recommendations are being proposed in order to produce xylitol

1) Addition of parameter

   For the Parameters that have been studied in this experiment are effect of time of hydrolysis, effect of pH and effect of amount of hydrolysate to production of xylitol. For the future study, additional parameter can be added such as effect of temperature, inhibition of material towards xylitol production and agitation speed effect to the fermentation process to produce xylitol.

2) Using other type of *Candida* strain

The other type of *Candida* strain can be use in order to determine the production of xylitol. The production of other strain of *Candida* might yield different amount of xylitol produced. *C. Boidinii C. Tropicalis C. Guillermondii C. Parapsilosis* are the type of other Candida strain that can be use (Ikeuchi *et al.,* 1999)

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APPENDICES

Calculation of concentration for each parameter was by using y = 0.0221x

1)Effect of time of hydrolysis to production of xylitol

y	x=y/0.0221
3.22	145.7013575
3.26	147.5113122
3.25	147.0588235
3.15	142.5339367
3.14	142.081448

Time (minutes)	Concentration (g/L)
30	145.70
60	147.51
90	147.06
120	142.53
150	142.08

2)Effect of pH to production of xylitol

y	x=y/0.0221
2.73	123.5294118
2.97	134.3891403
2.07	93.66515837
2.06	93.21266968
2.23	100.9049774

pH	Concentration (g/L)
1	123.53
2	134.39
3	93.67
4	93.21
5	100.9

3)Effect of amount of hydrolysate to production of xylitol

y	x=y/0.0221
2.27	102.7149321
2.21	100
2.16	97.73755656
2.15	97.28506787
2.4	108.5972851

Hydrolysate (mL)	Concentration (g/L)
10	97.28
20	100.00
30	97.74
40	102.71
50	108.59